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Treatment with a glycosaminoglycan formulation ameliorates experimental diabetic nephropathy

GIOVANNI GAMBARO, ANNA P. VENTURINI, DOUGLAS M. NOONAN, WALTER FRIES, GIULIANA RE, SPIRIDIONE GARBISA, CARLA MILANESI, ALESSANDRO PESARINI, ARTURO BORSATTI, EGIDIO MARCHI, and BRUNO BAGGIO

Institute of Internal Medicine and Division of Nephrology, Department of Biology, University of Padua, Padua; Alfa Wassermann Research Division, Bologna; and Cancer National Institute, Genoa, Italy

Treatment with a glycosaminoglycan formulation ameliorates experimental diabetic nephropathy. Previous studies have indicated that administration of glycosaminoglycans can prevent some of the morphological and physiological alterations which occur in experimental diabetic nephropathy. The aims of this study were to further elucidate the effect of these drugs on glomerular basement membrane permeability by dextran clearance studies, to test the ability of glycosaminoglycans to revert established diabetic nephropathy and to examine the effect of glycosaminoglycans on renal extracellular matrix synthesis. Five groups of Sprague-Dawley rats were studied for 12 months: two control groups (treated or untreated non-diabetic), three streptozotocin diabetic animal groups, two of which received a glycosaminoglycan formulation, one from the induction of diabetes and the other after the fifth month of diabetes. At five months the ^{35}S-sulfate glomerular incorporation, albuminuria, glomerular basement membrane thickness and anionic charge density were determined. At 12 months albuminuria, renal collagen IV and perlecan mRNA levels, anionic and neutral dextran clearances, glomerular basement membrane morphometry, and mesangial cell proliferation were evaluated. We demonstrate that long-term administration of glycosaminoglycans prevents renal morphological and functional alterations in diabetic rats and appears to revert established diabetic renal lesions. Glycosaminoglycan administration modified renal matrix composition by the normalization of collagen gene expression and increasing glomerular ^{35}S-sulfate incorporation.

We have previously reported that administration of glycosaminoglycans (GAGs) chronically from the onset of diabetes prevented glomerular basement membrane (GBM) thickening, glomerular anionic charge reduction, as well as the onset of albuminuria in experimental diabetic nephropathy without affecting glomerular filtration rate and metabolic control of the disease [1]. It has been shown that these drugs, irrespective of their anticoagulant activity, are also capable of slowing down the progression to uremia in rats with subtotal renal ablation [2, 3] and to ameliorate the nephropathy induced by chronic administration of puromycin [4], or by habu snake venom [5]. The exact mechanism of this "protective" effect of GAGs in experimental diabetic and other models of nephropathy is not known, though several hypotheses have been formulated: modulation of the coagulative cascade putatively involved in the pathogenesis of the glomerulopathy [6], down-regulation of several proteases [4], mechanical restoration of GBM anionic charges [2], and antimitogenic activity [5]. We have suggested as a plausible hypothesis that GAGs affect matrix synthesis by glomerular cells [1]. This study was designed: (1) to further elucidate the effect of these drugs on GBM permeability by dextran clearance studies; (2) to verify the ability of GAGs to revert established diabetic nephropathy; and (3) to examine the ability of GAGs to effect renal matrix synthesis by ^{35}S-sulfate incorporation and steady-state mRNA analysis for collagen IV and perlecan. These parameters were chosen as: (1) ^{35}S-sulfate incorporation effectively mirrors sulfated glycosaminoglycan synthesis due to the high incorporation of free sulfate into glycosaminoglycans, and (b) collagen IV and the heparan sulfate proteoglycan perlecan are the major components of the GBM known to be consistently affected by experimental nephropathy [7–10].

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Methods

Treatment protocol

Diabetes was induced in 40 male Sprague-Dawley rats (Charles River, Como, Italy) aged six to seven weeks (body wt ranging between 225 and 250 g) by intravenous administration of 35 mg/kg body wt streptozotocin (STZ) (Sigma Chemical Co., St. Louis, Missouri, USA) in a citrate buffer (pH 4.5). Fifteen diabetic rats served as diabetic controls (D), and starting immediately after diabetes induction received 2 ml/kg/day of saline solution s.c. five days weekly throughout the study. To another 15 rats with the same time schedule, a chemically modified fast-moving heparin (mH) (α-90-347, Alfa Wassermann SpA, Bologna, Italy) was administered at 15 mg/kg body wt/day subcutaneously (preventive protocol, D/mH/PP). The same drug was administered as above to the remaining 10 animals starting from the fifth month (curative protocol, D/mH/CP); until this time they received saline as the control animals above. The chemical structure of this drug derives from heparin, and is a glycosaminoglycan sulfate chain in which the 2-O-sulfate iduronic acid is converted in the galacturonic acid moiety. The molecular mass of mH is 10.4 kD, and the sulphate/carboxylic ratio is 1.35; the compound has a very low anticoagulant activity (110.2 μg/ml is necessary to induce a doubling of the aPTT time) [11]. The dosage was based on an equivalence for

antithrombotic activity ED_{50} with the drugs used previously [1]. As non-diabetic controls, two healthy male animal groups were utilized, one of which (C) consisted of 15 rats that received saline solution as above. The other control group was 10 animals treated with mH on the same schedule as the preventive protocol (C/mH). Throughout the study, all rat groups were fed a standard diet containing 20% protein (Altromin-R, Rieper, Bolzano, Italy). Diabetes mellitus was successfully induced in all STZ treated rats. No insulin treatment was given. During the 12 month follow-up, one rat in the D/mH/PP, two rats in both the C and D/mH/CP groups, and three rats in the D group died from infectious diseases. No rats had hemorrhagic disorders. Before diabetes induction, after five months, and at the end of the 12 month follow-up, the rats were weighed and transferred to individual metabolic cages for 24 hours for urine collection. Blood was withdrawn only during the conclusive urine collection, 24 hours after the last drug/saline administration. Creatinine was evaluated by the creatininase method and glucose by glucose oxidase. Albuminuria was assayed by the simple immunodiffusion technique using a rabbit anti-rat albumin antiserum and rat albumin as standard (USB Immunochemicals, Cleveland, Ohio, USA).

Fifth month analyses

At the fifth month, four randomly selected animals in the C, D and D/mH/PP groups were administered twice intraperitoneally with 1.0 mCi/100 g body wt of sodium $^{35}SO_4^=$ (462 mCi/mMol, NEN, Du Pont de Nemoirs, Dreieich, Germany). The injections (0.5 ml) were given at 3.5 hour intervals according to Beavan et al [12]. Three and one-half hours after the second injection animals were anesthetized with ketamine. Right kidneys were excised while the left kidney was used for an *in vivo* perfusion with a solution containing 0.2% ruthenium red (RR) (Carlo Erba, Milano, Italy) in Karnovsky's aldehyde fixative at a flow rate of 4 to 5 ml/min, with a perfusion pressure ranging from 90 to 100 mm Hg for five minutes as previously described [1]. The animals were then killed by exsanguination with cardiac puncture following anesthesia. Small tissue specimens were cut from the renal cortex of the upper pole of both kidneys and fixed for 20 hours at room temperature in Karnovsky's fixative (the right kidney) or in the same RR fixative mixture that had been used for perfusion (the left kidney). After appropriate rinsing and post-fixation, the specimens were dehydrated in ascending ethanols and embedded in Epon 812. Thick sections from right kidney cortices were cut and processed for autoradiography.

Twelfth month analyses

At the end of the follow-up, after 12 months from diabetes induction, all rats except five randomly selected animals from each group (C, C/mH, D, D/mH/PP, D/mH/CP) were sacrificed by exsanguination following ketamine anesthesia. Their right kidneys were excised, rapidly removed from the surrounding perirenal fat and transversely sectioned. Cortex from the lower pole was dissected and quick frozen in liquid nitrogen and stored at −80°C for mRNA studies. The upper pole was formalin-fixed, paraffin-embedded and sectioned for PCNA (proliferating cell nuclear antigen) immunohistochemistry. Twenty-four to 30 hours after the last s.c. drug/placebo administration, the five spared animals per group were anesthetized with ketamine and placed on a constant temperature table at 37°C. Heparinized polyethylene tubing was inserted into the left femoral vein for infusion of inulin, neutral and anionic dextrans, and in the abdominal aorta with the tip at the left renal artery level for *in vivo* renal perfusion, and in the left ureter for urine collection. Then, the right kidney was excised and processed as above for mRNA studies and PCNA immunohistochemistry. A saline (0.9% NaCl) solution containing 10 μCi/ml ^{14}C-inulin (NEN), 100 μCi/ml neutral ^{3}H-dextrans (NEN), and 10 μCI/ml anionic ^{125}I-dextrans was infused as a 0.6 ml bolus followed by a continuous intravenous infusion at a rate of 0.067 ml/min. Blood samples (0.5 ml) were drawn from the abdominal aorta at the beginning and end of the 30-minute urine collection period and mixed in equal volumes in order to average plasma concentrations of ^{3}H-dextrans, ^{125}I-dextrans, ^{14}C-inulin. Glomerular filtration rate (GFR) and dextran clearances were calculated by the standard formula. For the former, plasmatic and urinary values were obtained by summing up ^{14}C-inulin counts from all the correspondent chromatographic samples (see below). At the end of the clearance study, the left kidney was *in vivo* perfused with 0.2% RR in Karnovsky's aldehyde fixative as described above. The animals were then killed. Tissue specimens from the renal cortex of the upper pole were fixed and embedded in Epon 812 as described above for electron microscopy.

Autoradiography

Kidney sections were coated with Ilford K5 emulsion (Ilford, Mobberley, UK) at 43°C, and exposed for nine weeks in the presence of silica gel, in the dark, at 4°C. At the end of the exposure period the specimens were allowed to warm to 20°C and then developed without agitation. Development was stopped after three minutes using an acid step bath and the preparations were fixed in Agefix (Agfa Gevaert, Leverkusen, Germany) for 15 minutes. After being washed in running tap water, they were stained in 1% toluidine blue plus 1% borax solution for few seconds. Micrographs were prepared using a Leitz light microscope (Wetzlar, Germany).

Steady-state mRNA analysis

Total RNA was isolated from the frozen cortex by the method of Chomczynski and Sacchi [13]. The frozen material was immediately dispersed in the lysis buffer to limit the possibility of degradation. RNA yields were determined spectrophotometrically, and all samples used had a 260/280 nm ratio of 1.9 or greater. Dot blots on nitrocellulose membranes of serial dilutions of the RNA were made using a Schleicher and Schuell Minifold apparatus as follows: 40 μg of total RNA were adjusted to 10% formaldehyde in 10× sodium-sodium citrate (SSC) (20× SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.0). Serial dilutions (1:1) were then made with an equal volume of the same buffer containing tRNA as carrier, mixing, and removal of 1 volume for the following dilution. The serial dilutions (20, 10, 5 and 2.5 μg), were then vacuum blotted onto nitrocellulose (Schleicher and Schuell) equilibrated in 20× SSC. The wells were rinsed with 20× SSC, the blots removed and UV cross linked in a Stratalinker apparatus (Stratagene, La Jolla, California, USA) while still wet. Clone 16 encodes a portion of murine perlecan [14] which was found to cross-hybridize well with rat RNA. A Pst I fragment was isolated from the plasmid p-CIV-1-c87 (supplied by Dr. Steven Ledbetter, Upjohn Co., Kalamazoo, Michigan, USA) which contains the 5′ end of the α1 chain of collagen IV cDNA. As a control, a 0.6 kb Pst I-Eco RI fragment containing a portion of the sequence of cyclophillin, a "housekeeping" gene, was used. These cDNA

clones were radiolabeled by random priming (Boheringer Mannheim, Mannheim, Germany) and hybridized with the dot blots. The hybridizations and washes were performed under stringent conditions as previously described [15]. Blots were then exposed to Kodak X-Omat AR X-ray film. For reprobing, blots were stripped by incubating at 95°C in 0.02× SSC 0.2% SDS. Autoradiograms were quantitated by scanning on an LKB Ultroscan peak for each dot. The data from each dilution were then plotted to observe the linear portions of each series. Only samples which had a correlation coefficient greater than 0.95 in their linear range (minimum of 3 data points) were used. As the 10 μg point fell within the linear range for all samples, this point was used for all calculations. Data were expressed in optical density units (OD).

Morphometric analysis

Morphometric analysis on electron microscopic images was carried out blind on six glomeruli, for a total of 36 to 72 randomly selected GBM areas (final magnification ×40000) in each case. By means of a semiautomatic procedure with an image analyzer (Ibas 2000 Kontron, Carl Zeiss, Yena, Germany), the GBM thickness and the density of anionic charges stained by RR in the lamina rara externa were determined. Values were expressed as the number of RR precipitates per 1000 nm of GBM linear length. The morphometry of autoradiographic specimens was carried on with the same apparatus on 5 to 8 glomeruli per animal, 4 to 7 sections/glomerulus (final magnification ×1000) by mean of an automatic segmentation procedure. Their number was determined based on the grey value of the silver grain precipitates.

PCNA study

Immediately adjacent sections obtained from paraffin-embedded kidney specimens were evaluated blindly if they contained at least 20 glomeruli each. They were processed with a murine monoclonal antibody against human PCNA (Dako, Denmark) (avidin-biotin immunoperoxidase) and, for identification of mesangial cells, with a murine monoclonal antibody raised against muscle specific actin isoforms (HHF-35, Enzo Biochemicals, New Jersey, USA) [16] (avidin-biotin-immunoperoxidase). The latter antibody should be specific for proliferating mesangial cells, since α-actin is expressed only by proliferating mesangial cells and not quiescent mesangium as recently demonstrated [17]. For all specimens the negative control consisted of substituting the primary antibody with nonimmune murine serum. Proliferation was expressed as the numbers of nuclei per glomerular cross section that were positive for PCNA. The percentage of PCNA-positive nuclei and HHF-35-positive cells was calculated for 20 adjacent glomeruli in each animal.

Preparation of radiolabeled dextrans

Tritium radiolabeling of neutral dextrans having an average mol wt 40 kD (Sigma) was performed by NEN with tritium by oxidation with periodate, followed by reduction with tritiated sodium borohydrate according to Chang et al [18]. This same neutral dextran was sulfated to obtain an anionic compound using the method of Woods and Mora [19]. Briefly, 40 ml anhydrous pyridine were placed in a flask, and 10 ml of chlorosulfonic acid were added drop wise with vigorous stirring keeping temperature below 0°C. Thereafter, the reaction mixture was brought to 50°C, and 5 g of neutral dextran added. The reaction mixture was maintained at 60°C with stirring for five hours, and was then neutralized with sodium hydroxide to pH 7.0. The dextran sulfate was precipitated from the solution with ethanol, dialyzed against deionized distilled water, and lyophilized. Chemical analysis of the product indicated a sulfate molar ratio of 1.65 and no depolymerization. ^{125}Iodine radiolabeling of the sulfated dextran was performed according to Yoshioka et al [20]. Five hundred milligrams of dextran sulfate were then dissolved in 12.5 ml of a 0.2 M $NaIO_4$ solution and kept 24 hours in the dark at room temperature. Thereafter, dextran oxidation was stopped with 1 ml ethylene glycol. The oxidized dextran solution was dialyzed against repeated changes of distilled water and added to a solution containing 0. 2 M borate buffer (pH 9.0) and 0.1 M tyramine in 0.1 N HCl, mixed, and kept at room temperature for two hours. The mixture of oxidized dextrans was reduced with 1% $NaBH_4$, and the excess of $NaBH_4$ was neutralized by the addition of acetone. After dialysis, the dialysate was centrifuged at 8000 rpm for 40 minutes, and the supernatant was then lyophilized. The dextran was then purified by precipitating it in a solution of sodium acetate in ethanol, filtering and washing in absolute ethanol, and finally drying under vacuum at 40°C. This purified dextran was solubilized in a phosphate buffer (pH 7.4) and labeled with 20 μl ^{125}I (100 μCi) by incubation with 5 units IODO-Bead (Pierce, Rockford, Illinois, USA) for 15 minutes at 4°C. After labeling, ^{125}I-dextran was separated from free ^{125}I by chromatography through a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) equilibrated with phosphate buffer (pH 7.4). ^{125}I-dextran was collected and total radioactivity was counted in a gamma counter (Beckman Instruments, Irvine, California, USA). After filtration (0.45 μm pore size filter, Millipore, Bedford, Massachusetts, USA) the solution of iodinated dextran (specific activity 17 μCi/mg) was stored at 4°C in sterile vials.

Chromatographic procedures

Plasma and urine samples were assayed for ^{14}C-inulin, as described below, to determine GFR and then separated on a molecular sieve column for ^{3}H-neutral dextrans and ^{125}I-anionic dextran content by high performance liquid chromatography (Beckman Model 344) using a 7.5 × 360 mm Spherogel TSK 2000 column (Beckman) with a 7.5 × 75 mm Sepharose Guard precolumn (Beckman). PBS was used to equilibrate the chromatographic system and to dilute the samples (up to 500 μl) before sieving at a flow rate of 1 ml/min; the absorbance of the eluate was monitored at 214 nm. Fractions of 1 ml were directly counted for ^{125}I (anionic dextran) radioactivity in a gamma counted (Beckman), and then dissolved in 5 ml of Ultima Gold (Packard, Groningen, The Netherland) and counted for ^{3}H (neutral dextran) in a LS1801 Beckman scintillation counter using 0-400 and 400-670 adjusted quenching windows. The column was calibrated using bovine serum albumin and ribonuclease A (Pharmacia).

Statistical analyses

One-way analysis of variance (ANOVA) was used for glomerular filtration rate (creatinine clearance), dextran clearances, albuminuria, type IV collagen mRNA and perlecan mRNA, and two-way ANOVA for 5 and 12 month morphometric parameters, and autoradiographic data. The latter analyses enabled evaluation of the effect of both treatment and animal variability on charge density, GBM thickness, and ^{35}S-sulfate glomerular incorporation. After ANOVA, specific hypotheses regarding differences in morphometric parameters among groups were tested by contrasts

Table 1. Parameters evaluated at 5 and 12 months of follow-up in control (C) and diabetic (D) rats treated with a fast moving heparin with a preventive protocol (C/mH, D/mH/PP), and in diabetic rats with a curative schedule (D/mH/CP)

	Time *months*	C	C/mH	D	D/mH/PP	D/mH/CP
Body wt *g*	12	798 ± 38 (9)	721 ± 28 (10)	385 ± 21 (8)	369 ± 24 (10)	390 ± 27 (8)
Glycemia *mg/dl*	12	112.3 ± 4.1 (9)	129.8 ± 3.9 (10)	732.3 ± 56.2 (8)	838.7 ± 68.2 (10)	791.0 ± 49.5 (8)
GFR *ml/min/kg body wt*	12	7.21 ± 0.84 (9)	8.01 ± 0.92 (10)	10.20 ± 1.89 (8)	9.76 ± 2.01 (10)	9.43 ± 1.64 (8)
Albuminuria *mg/24 hr*	5	3.5 ± 1.3 (15)	—	18.2 ± 4.7 (13)	7.0 ± 1.8 (15)	—
	12	20.9 ± 7.5 (9)	13.4 ± 4.6 (10)	116.0 ± 31.9 (8)	38.8 ± 8.5 (10)	18.5 ± 2.4 (8)
GMB thickness *nm*	5	263 ± 6 (4)	—	297 ± 7 (4)	—	—
	12	460 ± 12 (4)	420 ± 21 (4)	536 ± 11 (4)	420 ± 12 (4)	415 ± 9 (4)
GBM charge density *points/1000 nm*	5	44.9 ± 1.4 (4)	—	39.7 ± 1.0 (4)	—	—
	12	42.4 ± 1.2 (4)	52.3 ± 1.7 (4)	32.2 ± 0.6 (4)	45.2 ± 1.3 (4)	49.4 ± 2.3 (4)
^{35}S incorporation Ag^{2+} *grains/1000* μm^2	5	62 ± 4 (4)	—	20 ± 5 (4)	37 ± 3 (4)	—
Collagen IV mRNA (*OD*)	12	0.49 ± 0.06 (5)	0.58 ± 0.06 (7)	0.77 ± 0.07 (5)	0.50 ± 0.05 (7)	0.51 ± 0.05 (5)
Perlecan mRNA (*OD*)	12	0.39 ± 0.06 (5)	0.17 ± 0.02 (7)	0.31 ± 0.02 (5)	0.21 ± 0.02 (7)	0.23 ± 0.04 (5)

Data are mean ± SEM (number of rats).

(linear combinations of means). Bonferroni's test for multiple comparisons was applied to GFR and albuminuria values. The Kruskal-Wallis non-parametric test was employed to compare the average percentages of PCNA- and HHF-35-positive cells.

Results

Mean values and standard errors of the parameters concerning glycemia, body weight, creatinine clearance, GBM thickness, GBM charge density, ^{35}S-sulfate incorporation, albuminuria, type IV collagen mRNA and perlecan mRNA are reported in Table 1. Metabolic control, with reference to glycemia and body weight, was similar in all diabetic groups. Albeit diabetic groups had a trend towards higher GFR values with respect to healthy rats, the differences were not significant ($F = 0.67$, Table 1).

After five months, diabetic animals showed ultrastructural and functional evidence of renal involvement; in fact albuminuria was mildly increased ($F = 7.18$; $P < 0.002$; Fig. 1), as well as GBM thickness ($F = 4.69$; $P < 0.02$; Fig. 2), while GBM charge density was decreased ($F = 4.02$; $P < 0.02$; Fig. 3). After 12 months, the urinary albumin excretion was markedly increased in untreated diabetic rats ($F = 8.61$; $P = 0.000$). However, there was no significant difference between control and either the C, C/mH, D/mH/PP or D/mH/CP groups or between the D/mH/PP and D/mH/CP groups for urinary albumin excretion (Table 1, Fig. 1). There was no difference in neutral dextran fractional clearances among the C, D, and D/mH/PP groups over the entire range of molecular radii studied (24 to 50 Å); on the contrary, diabetic animals had a higher anionic dextran clearances for radii greater than 43 Å (Fig. 4), but there was no significant difference between the D/mH/PP and the C group for radii over 45 Å (Fig. 4).

At 12 months, GBM thickness was greater in the D group as compared to healthy control animals, to the C/mH group, and to the D/mH/PP and D/mH/CP groups ($F = 18.55$; $P = 0.000$). There were no significant differences in GBM thickness between the control and the C/mH, and D/mH/PP, and the D/mH/CP groups (Table 1, Fig. 2). The GBM charge density was significantly lower in D animals ($F = 20.79$; $P = 0.000$) in respect to all other groups. C/mH group had significantly higher charge density also in respect to the control and the D/mH/PP groups ($P < 0.001$ and $P < 0.01$, respectively; Table 1, Fig. 3). The steady state level of perlecan mRNA in diabetic animals (D) was not statistically different from that of healthy animals (C); however, it was reduced in all treated groups (C/mH, D/mH/PP, D/mH/CP) ($F = 8.58$; $P = 0.000$) with respect to untreated control animals (Table 1, Fig. 5; $P < 0.001$, < 0.005, < 0.005, respectively). Type IV collagen mRNA in diabetic rats (D) was significantly increased with respect to all other groups ($F = 5.01$; $P < 0.002$), but there was no significant difference between those other groups (Table 1, Fig. 6). Autoradiography disclosed a reduced glomerular ^{35}S-sulfate incorporation in the D group ($F = 28.4$; $P = 0.000$) with respect to both C ($P < 0.001$) and D/mH/PP ($P < 0.02$) groups. The D/mH/PP group also showed lower ^{35}S-sulfate incorporation than in controls ($P < 0.01$) (Table 1, Fig. 7). No difference emerged within groups for GBM thickness, GBM charge density and ^{35}S-sulfate incorporation ($F = 1.90$, 0.98, 2.31, respectively).

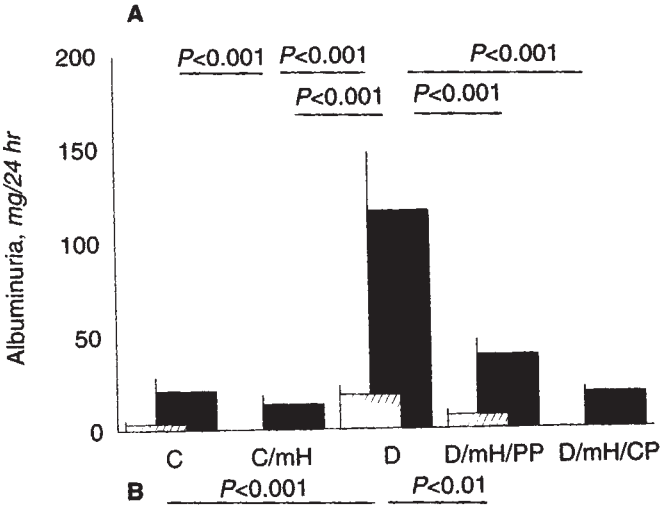

Fig. 1. *Albumin urinary excretion (mean and SEM) in control rats (C), in diabetic rats (D), in healthy and diabetic rats treated with a fast moving heparin with the preventive protocol (C/mH and D/mH/PP, respectively), and in diabetic rats treated with the curative (D/mH/CP) schedule, at 5 (shaded columns) and 12 months ($F = 7.18$; $P = 0.002$ and $F = 8.61$; $P = 0.000$ respectively).* **A.** Significancy bars refer to 12 month values, **B** are 5 month data.

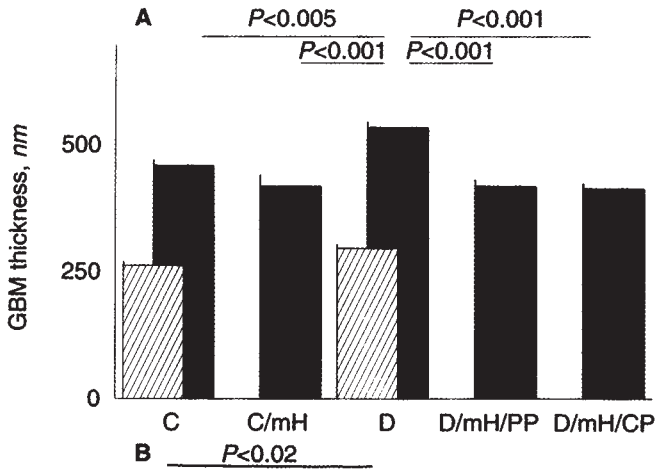

Fig. 2. *Glomerular basement membrane thickness (mean and SEM) in control rats (C), in diabetic rats (D), in healthy and diabetic rats treated with a fast moving heparin with the preventive protocol (C/mH and D/mH/PP, respectively), and in diabetic rats treated with the curative (D/mH/CP) schedule, at 5 (shaded columns) and 12 months ($F = 4.69$; $P < 0.02$ and $F = 18.55$; $P = 0.000$ respectively).* **A.** Significancy bars refer to 12 month values, **B** are 5 month data.

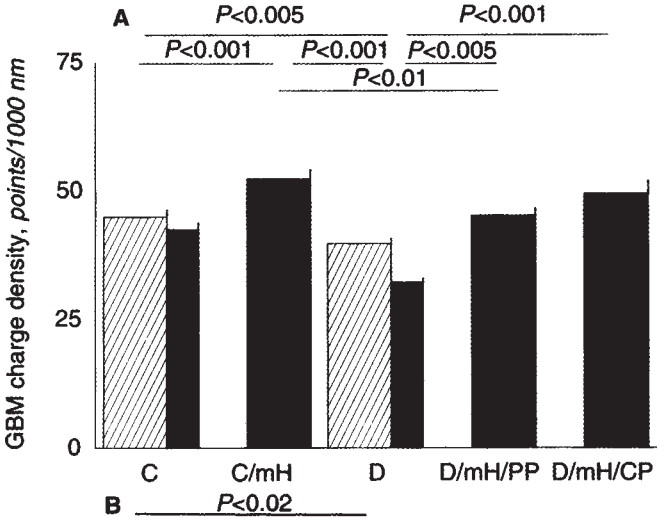

Fig. 3. *Anionic charge density (mean and SEM) in the glomerular basement membrane in control rats (C), in diabetic rats (D), in healthy and diabetic rats treated with a fast moving heparin with the preventive protocol (C/mH and D/mH/PP, respectively), and in diabetic rats treated with the curative (D/mH/CP) schedule, at 5 (shaded columns) and 12 months ($F = 4.02$; $P < 0.02$ and $F = 20.79$; $P < 0.001$ respectively).* **A.** Significancy bars refer to 12 month values, **B** are 5 month data.

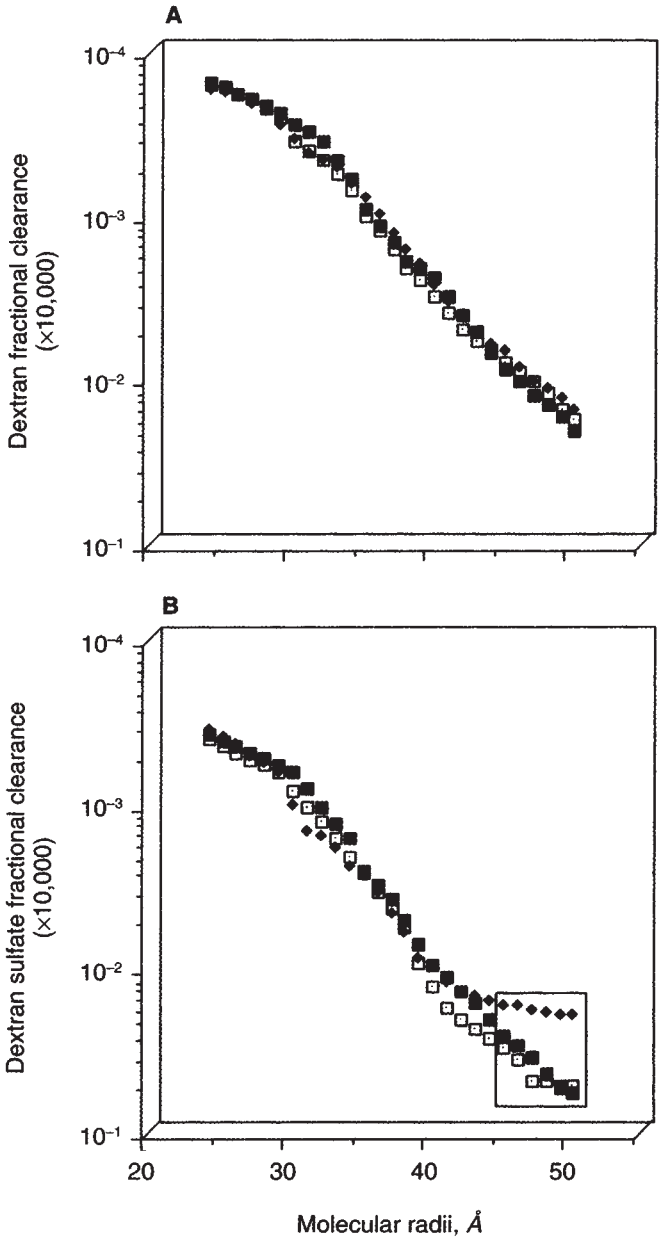

Fig. 4. *Fractional clearances of neutral* **A** *and anionic* **B** *dextrans in control (C, □), in diabetic (D, ◆) and in heparin treated diabetic (D/mH/PP, ■) rats at 12 months.* SEM was lower than 10% of mean values for all the evaluated molecular radii. Statistically significant different anionic dextran clearances between C and D/mH/PP vs. D animals are framed by the square.

Finally, on the average, less than 1 PCNA-positive cell and less than 1 HHF-35-stainable cell per animal were observed in all groups, without showing any significant differences ($P = 0.28$ and 0.45, respectively).

Discussion

This study confirms previous observations that chronic administration of glycosaminoglycans is capable of preventing morphological and functional abnormalities in GBM induced by diabetes [1]. In fact, administration of mH-glycosaminoglycans in diabetic rats was capable of preventing GBM thickening (Fig. 2) and the reduction in GBM anionic charges (Fig. 3), while maintaining normal urinary albumin excretion (Fig. 1). Dextran clearance analyses unequivocally demonstrate that glycosaminoglycans can prevent alterations in GBM permeability, as suggested by their ability to reduce albuminuria [1, present data]. In fact, the charge

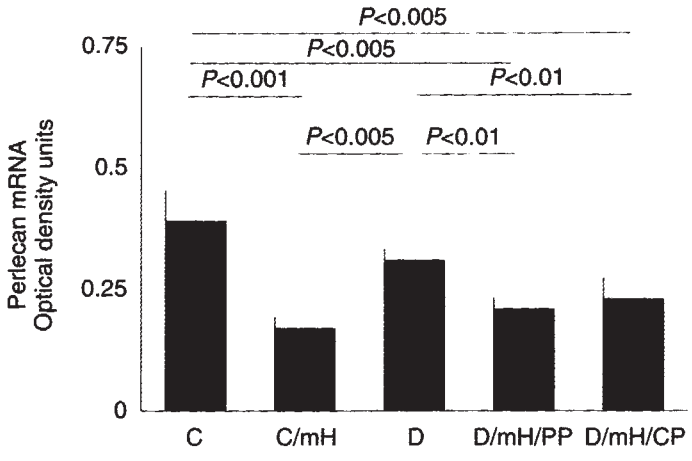

Fig. 5. *Perlecan mRNA levels (mean and SEM) in the renal cortex at 12 months in control rats (C), in diabetic rats (D), in healthy and diabetic rats treated with a fast moving heparin with the preventive protocol (C/mH and D/mH/PP, respectively), and in diabetic rats treated with the curative (D/mH/CP) schedule.* F = 8.58; $P = 0.000$.

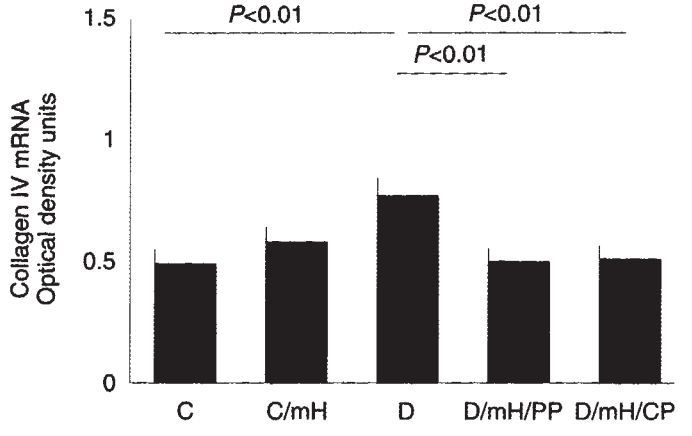

Fig. 6. *Type IV collagen mRNA (mean and SEM) levels at 12 months in control rats (C), in diabetic rats (D), in healthy and diabetic rats treated with a fast moving heparin with the preventive protocol (C/mH and D/mH/PP, respectively), and in diabetic rats treated with the curative (D/mH/CP) schedule.* F = 5.01; $P < 0.002$.

permselectivity in heparin-treated diabetic rats was similar to the healthy control rats, whereas the untreated diabetic animal had abnormally high clearances over 45 Å (Fig. 4).

The dextran glomerular permeability data seem to mirror previous observations in humans that GBM charge selectivity to macromolecules is compromised in early diabetic nephropathy and precedes a size selectivity derangement [21–23]. It should be noted that the correction of GBM permeability parallels the conservation of GBM morphology, that is, normal GBM width and anionic charge density, thus providing evidence that abnormal permselectivity is directly related to these ultrastructural alterations.

Furthermore, here we show that it is possible to reverse established GBM diabetic lesions, leading to a normalization of GBM morphology and function. After five months the diabetic animals had mild, albeit significant, signs of nephropathy which were reversed following seven months of mH therapy. The ability of GAGs to reverse GBM abnormalities is unique in that previous studies have demonstrated that pancreatic islet transplantation was not able to reverse changes in GBM thickness [24, 25]. This paradox may be due to the prolonged half-life of some cellular changes induced by diabetes [26–28]. It is possible that these heparin species affect different points in the cellular metabolism than diabetes, "forcing" a correction of matrix synthesis which does not occur with the normalization of glycemia or insulinemia alone. Certainly the ability of mH-glycosaminoglycans to modulate matrix/GBM synthesis does not exclusively depend on cellular changes induced by diabetes, since heparin treatment modulated GBM production in the healthy rats as well. In fact, mH in healthy animals induced an increase of GBM charge density and a reduction of perlecan mRNA steady-state levels (Figs. 3, 5).

The mechanism of GAG activity on diabetic nephropathy is not clear. However, the anticoagulant properties, hemodynamic effects, down-regulation of several proteases, a putative activity on non-enzymatic glycation, and mechanical restoration of glomerular charge by these compounds seem insufficient to explain the full picture of the preventive effects of GAGs in the above experimental models [reviewed in 1]. Furthermore, the possibility of an anorexant effect of heparin leading to a reduced protein intake and consequently to a diminished glomerular hyperfiltration seems unlikely. In fact, such an effect has never been described, and heparin treated animals did not significantly differ in weight in respect to their own controls.

The mH-glycosaminoglycan preparation results in material which is largely desulfated. Heparin maintains its activity in different experimental models of nephropathies irrespective of its sulfation ratio [3, 4]. The results we observed here with mH-heparin are very similar to those previously obtained with molecules with different sulfation, such as a low molecular weight heparin and a dermatan sulfate [1], confirming that even great variations in the sulfation ratio of the exogenous GAGs are not critical for the ability of these molecules to prevent or reverse nephropathic damage. Although an abnormal sulfate metabolism has been described in diabetic animals [27], the large independence of the activity from the degree of sulfation suggests that mere supply of sulfate does not explain the GAG effect on diabetic nephropathy.

The antiproliferative activity of GAGs or the possibility that these compounds modulate matrix/GBM synthesis give more likely explanations of their effects. The antimitogenic effect of GAGs on a number of cells is well known [29–32]. GAGs have also been shown to inhibit mesangial cell proliferation and prevent disease progression in the subtotal nephrectomized rat model [2, 3, 6, 33], in puromycin or habu snake-induced nephrosis of the rat [4, 5], and in an experimental mesangioproliferative glomerulonephritis [34]. The possibility that GAGs affect the proliferation of mesangial and other glomerular cells in diabetic nephropathy seems unlikely, since it is generally considered that mesangial expansion in diabetes is due to the extracellular matrix enlargement rather than to mesangial cell hyperplasia. Furthermore, we have demonstrated the lack of any glomerular proliferative wave in STZ diabetic rats 12 months after the induction of diabetes [35], and our present results show no difference between the treated and untreated diabetic and healthy groups concerning PCNA and HHF-35 stainable glomerular cells.

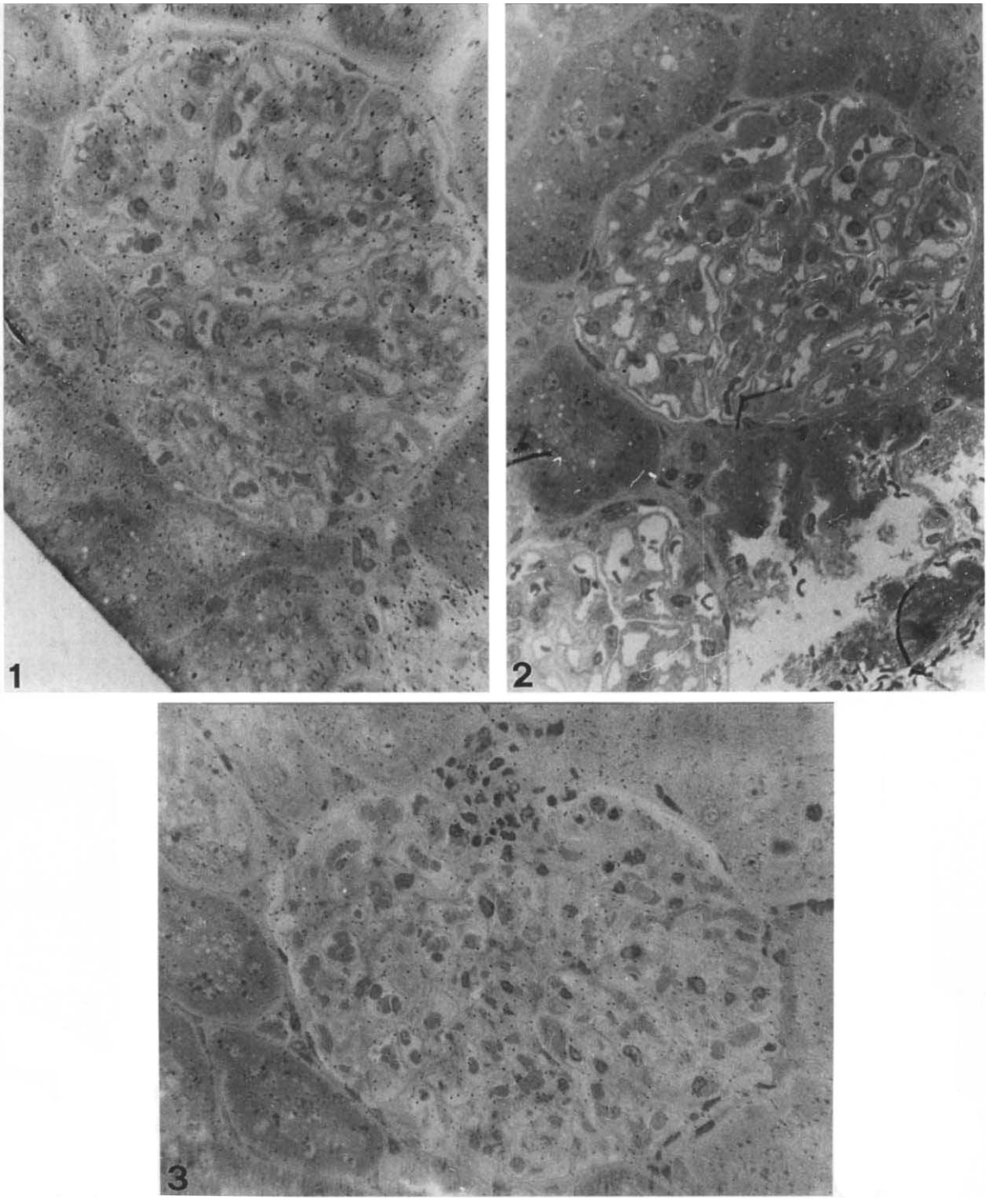

Fig. 7. *Autoradiographs showing ^{35}S-sulfate in glomerular areas.* Black points correspond to silver precipitates. Micrograph 1 is from a healthy rat, micrograph 2 from a diabetic animal, and micrograph 3 from a group D/mH/PP rat.

GAGs have been demonstrated to modify the synthesis of proteoglycans and of other matrix proteins in vascular smooth muscle and endothelial cells, influencing the synthesis and composition of the extracellular matrix [36–38]. We have hypothesized that these agents could have an effect on the synthesis of matrix/GBM constituents by glomerular cells, leading to the correction of some biochemical or cell functional abnormalities responsible for GBM thickening and glomerulosclerosis [1]. The results of the present study confirm the hypothesis that GAGs affect matrix synthesis *in vivo*. Heparin treatment, in fact, altered

type IV collagen (Fig. 6) and perlecan (Fig. 5) mRNA levels, and increased glomerular ^{35}S-sulfate incorporation (Fig. 7) in diabetic animals.

Metabolic studies in experimental models have provided evidence that enhanced production of collagen is a central event in the development of diabetic glomerular extracellular matrix abnormalities [39–41]. Increased immunohistochemical staining to type IV collagen in the expanded mesangium and GBM in diabetic nephropathy has been shown [42, 43], and in basement membranes of human diabetic tissues increased levels of type IV collagen have been observed [44]. In the KKAy mouse and in the WKY rat, two genetically transmitted models of NIDDM that demonstrate thickening of the glomerular basement membrane with duration of diabetes, as well as in the STZ-induced diabetic rats, steady state levels of mRNA encoding type IV collagen progressively increase [7–10], confirming our present results. The observation that both type IV collagen mRNA and protein are increased in the kidney of diabetic rats is consistent with increased transcription of the corresponding gene in diabetes, rather than with abnormal post-transcriptional processes or reduced collagen degradation. The reduced type IV collagen mRNA levels in the heparin treated diabetic animals probably reflect reduced collagen synthesis as supported by recent findings obtained in experimental mesangioproliferative glomerulonephritis where heparin inhibits the overproduction of extracellular matrix proteins, including type IV collagen [34]. However, in smooth muscle cells in culture, heparin in the presence of the endothelial cell growth factor appears to modulate the synthesis of collagen at a translational and/or post-translational level, since the mRNA steady-state concentrations were not significantly altered [36], and heparan sulfate alone, whose chemical structure is strictly related to heparin, increases type IV collagen synthesis in mesangial cells [45]. It is evident that a more complex situation may be present *in vivo* which leads to reduced collagen IV mRNA levels.

There was no difference in perlecan mRNA levels in D rats (Fig. 5) similar to that observed in the non-insulin dependent diabetes mouse model (KKAy) [7]. At first glance this is in contrast with the reduced GBM anionic charge density, as these charges correspond to heparan sulfate rich sites [46], and the coexistent albuminuria which is believed to reflect an abnormally-low GBM heparan sulfate content [47]. Some explanations of this apparent contradiction can be rationalized. It has been observed that the components of basement membrane interact in a highly specific manner with a very strict stoichiometry, and that the authentic architecture of the membrane (with consequent permeability characteristics) can be achieved only as long as the component precursors are secreted in correct concentrations [48, 49]. Thus abnormalities of the molar ratio between different components of GBM, that is, perlecan and type IV collagen, which are probable in light of our mRNA data, might have profound consequences on the supramolecular organization of GBM leading to the reduced anionic charge density observed in diabetes. Alternatively, the decrease of GBM anionic charge density in diabetic rats might be due to a reduced/abnormal heparan sulfate expression with normal perlecan core protein synthesis in the GBM as a consequence of post-transcriptional processes (that is, impaired synthesis, enhanced degradation, or reduced sulfation of the heparan sulfate carbohydrate chains). The latter hypothesis is supported by the observation that the normal perlecan mRNA levels in diabetic rats are in accordance with normal staining with monoclonal antibodies against the perlecan core protein in diabetic glomerulopathy, while the staining with anti-heparan sulfate side chain antibodies is reduced [50]. In addition, normal perlecan mRNA levels but reduced levels of N-deacetylase, a critical enzyme involved in heparan sulfate side chain sulfation, are found in short-term diabetes [51].

In both healthy and diabetic heparin-treated rats, a trend towards reduced perlecan steady-state mRNA levels appears which might be expression of a down-regulatory mechanism (Fig. 5). However, heparin also appears to have a regulatory activity at the level of translational or post-translational processing of proteoglycans. In mesangial cell cultures it increases heparan sulfate synthesis and ^{35}S-sulfate uptake [37, 52], and on endothelial cells it increases the synthesis and sulfation of heparan sulfate [38]. Thus, the ameliorated anionic charge density in the GBM in heparin treated animals (Fig. 3) may depend on the effect of these agents on sulfation or synthesis of the heparan sulfate side chains. The results of our ^{35}S-sulfate autoradiographic study support this concept (Fig. 7), in that while diabetic animals had a very low silver grain density (corresponding to decreased ^{35}S-sulfate incorporation) confirming previous observations [29], the heparin treated animals had an increased grain density which most likely reflects a stimulated sulfate/glycosaminoglycan metabolism. It is clear that these changes are not due to alterations in perlecan mRNA levels (reflecting core protein synthesis), as this is not changed in the diabetic untreated animals and is lowered in heparin treated animals. The increased ^{35}S-sulfate incorporation is more likely due to either increased side chain sulfation and/or increased synthesis of other proteoglycan species.

In conclusion, our study demonstrates that the favorable effect of glycosaminoglycans on GBM morphology and function in experimental diabetes depends on their activity on the synthesis of matrix/GBM molecules by glomerular resident cells.

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Reprint requests to Giovanni Gambaro, M.D., Ph.D., Istituto di Medicina Interna, Divisione di Nefrologia 1, Policlinico Universitario, via Giustiniani, 2, 35120 Padova, Italia.

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